Efficient Chemical Synthesis of CMP-Neu5Ac and CMP-(Neu5Acα2→8Neu5Ac)

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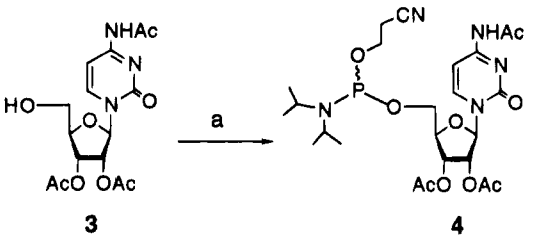
Cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), when acted upon by sialyltransferase, acts as a source of sialic acid in the synthesis of sialyl oligosaccharides.[1] The availability of CMP-Neu5Ac synthetase[2] via cloning technology allows for the synthesis of CMP-Neu5Ac analogues. These analogues often possess improved sialyl-donating properties. Among the Neu5Ac analogues which have already been enzymatically synthesized are those in which the 4, 5, 8, and 9 positions of Neu5Ac have been modified.[3] The Neu5Ac analogue with a large substitutent at the 9 position has been shown to be an especially good sialyl donor analogue.[3c] It is reasonable to assume that in this case the bulky substitutent is positioned away from the catalytic site of the sialyltransferase. Therefore, CMP-Neu5Ac analogues having a large substitution at the 7 or the 8 position would also be expected to act as efficient sialyl donors. Among the possible CMP-Neu5Ac analogues, we chose CMP-(Neu5Acα2→8Neu5Ac) (= CMP-Neu5Ac-dimer (**1**)) which could act as a sialyl donor for the enzymatic synthesis of the Neu5Acα2→8Neu5Acα2–3Gal or α2–6Gal units which occur in both glycolipids and glycoproteins. The reported synthetic methods[4] for the preparation of CMP-Neu5Ac did not provide the desired CMP-(Neu5Ac-dimer (**1**)), however.[5] Therefore, in order to synthesize this novel analogue, several problems had to be addressed. Since the reactivity of the quaternary carbon at the 2 position is poor when compared with that of Neu5Ac,[6] an efficient phosphitylating reaction between the Neu5Ac-dimer and the cytidine derivative was critical. In addition, the phosphorotriester

Scheme 1[a]



[a] Key: (a) 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, diisopropylamine, MeCN–DMF.

group in the protected CMP-Neu5Ac is prone to elimination as seen during the glycosylation reaction using glycosyl 1-phosphate.[7] Indeed, we found that the protected CMP-Neu5Ac has this same tendency for elimination. Therefore, an optimized deprotection method avoiding this unfavorable side reaction was also required. We developed a suitable synthetic model, and synthesis of CMP-Neu5Ac (**2**) was undertaken in order to solve these problems. In this report, we detail the most efficient synthetic route to CMP-Neu5Ac (**2**) yet published (52% overall yield based on methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate) and also the successful synthesis of the CMP-Neu5Ac-dimer (**1**).

Using the knowledge that CMP-Neu5Ac (**2**) is stable under strong basic conditions (pH > 11), we devised a synthetic route which employed acetyl protecting groups and 2-cyanoethyl *2′,3′-O,N*[4]-triacetylcytidin-5′-yl *N,N*-diisopropylphosphoroamidite (**4**) in the phosphitylating reaction (Scheme 1). To prepare the cytidine 5′-*O*-amidite **4**, introduction of an amidite group to the 5′-position of the cytidine derivative **3**[8] with 2-cyanoethyl *N,N,N′,N′*-tetraisopropyldiamidite in the presence of diisopropylamine and 1*H*-tetrazole afforded amidite **4** as a diastereomeric mixture (1:1) in 72% yield.

Coupling of pentaacetyl Neu5Ac **5**[9] with the amidite **4** in the presence of 1*H*-tetrazole provided the phosphite derivative **6** quantitatively (Scheme 2). Purification of the crude phosphite on a column of silica gel afforded pure **6** in 47% yield. Although the phosphite **6** was stable even in the crude state in several organic solvents for at least 1 week, this compound easily decomposed on silica gel and the decomposition rate was proportional to the quantity of silica gel. An improved purification procedure using Sephadex LH-20 (MeOH) gave pure phosphite **6** in 83% yield (1.6:1 diastereomeric mixture). The oxidation of phosphite **6** to the phosphate derivative **7** (1.6:1 diastereomeric mixture) was performed using *tert*-butyl hydroperoxide in 90% yield. De-*O*- and de-*N*-acetylation of phosphate **7** was attempted under several different conditions such as NaOMe (5 equiv) in MeOH, NH_4OH–MeOH (3:1), triethylamine–MeOH–H_2O, and Na_2CO_3 in MeOH–H_2O. However, these reaction conditions led to sialoside **8**[10] (α only) as the major compound along with exclusively minor CMP-Neu5Ac (**1**). Assuming that the sodium or ammonium cation assists the elimination of

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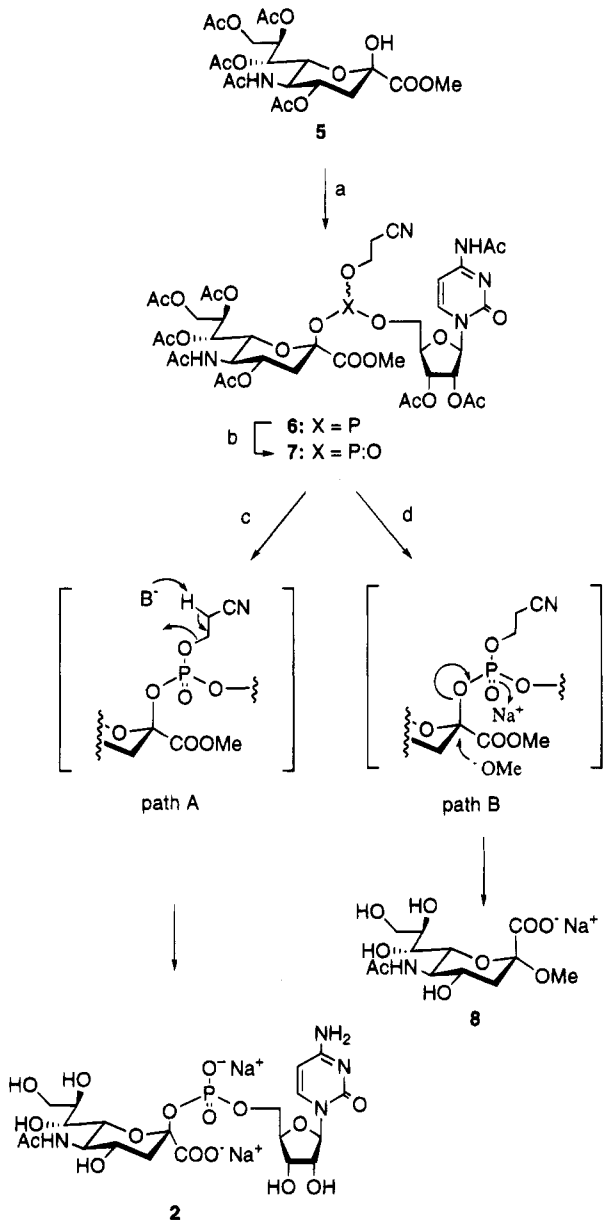
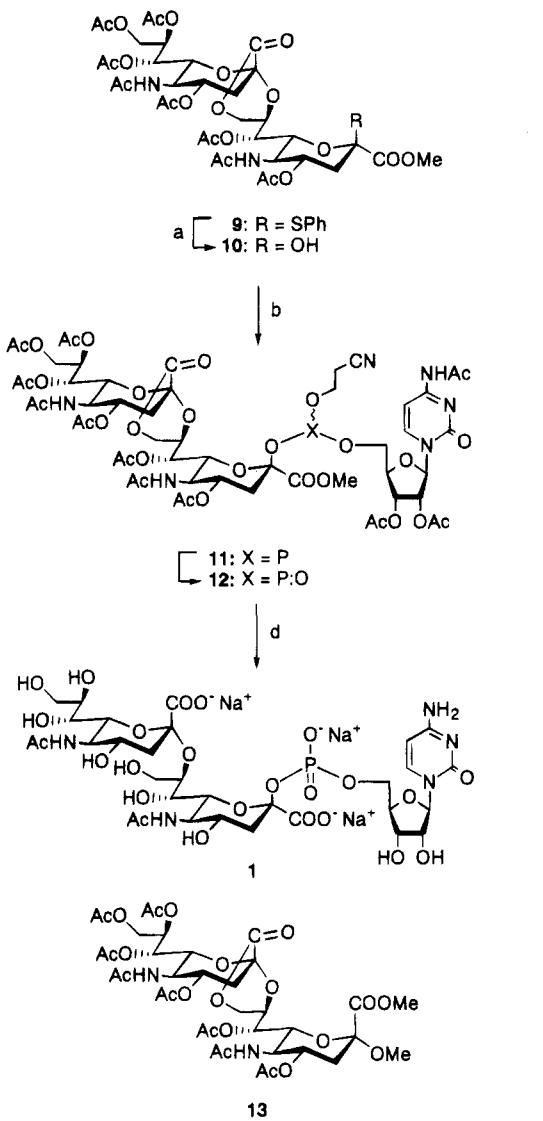
Scheme 2[a]



[a] Key: (a) 3 equiv of 4, 1*H*-tetrazole, MeCN, 83%; (b) t-BuOOH, MeCN, 90%; (c) condition a, 50 equiv of NaOMe, MeOH, H_2O, 75%; condition b, (1) DBU, THF; (2) NaOMe, MeOH–H_2O, 69%; (d) 5 equiv of NaOMe, MeOH, H_2O or NH_4OH–MeOH or triethylamine, MeOH–H_2O or Na_2CO_3, MeOH–H_2O.

the phosphorotriester group to give α-sialoside (path B), we hypothesized that if the cyanoethyl group was first removed, CMP-Neu5Ac **2** could be obtained due to the stability of the phosphorodiester (path A). Therefore, in order to abstract the α hydrogen of the cyanide, an excess of NaOMe (50 equiv) was used for the de-*O*- and de-*N*-acetylation. These conditions provided the desired CMP-Neu5Ac **2** in 75% yield. However, when the reaction was scaled up, the reproducibility was poor. Therefore, we investigated alternate conditions for this step. De-*O*-cyanoethylation with DBU in tetrahydrofuran and subsequent de-*O*- and de-*N*-acetylation with NaOMe proved to work quite well (69% yield), and the reproducibility of the deprotection step was greatly improved. The desired CMP-Neu5Ac **2** was obtained in 52% overall yield based on **5**.

Scheme 3[a]



[a] Key: (a) NBS, acetone–H_2O, 89%; (b) 7.8 equiv of 4, 1*H*-tetrazole, MeCN, 85%; (c) t-BuOOH, MeCN, 73%; (d) (1) DBU, THF; (2) NaOMe, MeOH–H_2O, 56%.

The same synthetic route developed for CMP-Neu5Ac (**2**) was used for the synthesis of CMP-Neu5Ac-dimer **1**. The Neu5Ac-dimer thioglycoside **9**[6] was treated with *N*-bromosuccinimide to give **10** (only β-configuration) in 89% yield (Scheme 3). This was followed by a series of reactions analogues to the previous synthesis to provide the CMP-Neu5Ac-dimer **1**[11] in 35% overall yield (based on **10**). During the oxidation of phosphite **11**, one of the diastereomers decomposed slightly. Purification of the phosphate **12** on a gel permeation column (Sephadex LH-20, MeOH) afforded the sialoside **13** (α only) quantitatively. Therefore, dichloromethane was used as the solvent for this gel permeation step.

The structures of the synthetic CMP-Neu5Ac (**2**) and CMP-Neu5Ac-dimer (**1**) were characterized from their ^1H and ^{31}P spectra (Table 1). The ^1H-NMR spectrum of the synthetic CMP-Neu5Ac (**2**) was in good agreement with

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(11) The CMP-Neu5Ac dimer is very labile to acid compared to CMP-Neu5Ac.

Table 1. ^1H and ^{31}PNMR Chemical Shifts[a]

	4	6	7	10	11	12	1[b]	13
Neu5Ac								
H-3ax		2.23-1.85	2.26-1.85	2.34-2.25	1.83 (12.0, 12.0)	1.66 (10.3, 10.3)	1.64 (12.1, 12.6)	2.22-2.19
		2.23-1.85	2.26-1.85	2.34-2.25	1.51 (11.5, 11.5)	1.41 (9.5, 9.5)	1.59 (2.7, 13.3, 13.3)	2.22-2.19
H-3eq		2.50 (4.4, 12.9)	2.99 (4.9, 13.7)	2.45 (5.6, 13.3)	2.60-2.25	2.92 (4.8, 13.8)	2.64 (4.3, 12.6)	2.63 (4.9, 13.0)
		2.48 (4.9, 12.8)	2.65 (4.8, 13.3)	2.34-2.25		2.60 (5.1, 13.1)	2.44 (4.7, 13.3)	2.46 (5.4, 13.5)
						2.39 (5.8, 13.7)		
NH		7.8 (8.6)	7.69-7.56	5.76 (10.1)	7.76 (8.7)	7.74 (9.4)		5.50-5.48
		6.87 (10.0)	7.69-7.56	5.43 (10.4)	6.82 (10.1)	7.37 (10.0)		5.50-5.48
Cytidine								
H-5	7.44 (7.6)	7.52 (7.5)	7.52 (7.5)		7.46 (7.5)	7.49-7.43	6.01 (7.5)	
	7.44 (7.6)	7.45 (7.5)	7.44 (7.5)		7.42 (7.5)	7.49-7.43		
H-6	8.26 (7.6)	8.07 (7.5)	7.69-7.56		7.84 (7.5)	7.56 (7.6)	7. 86 (7.5)	
	8.24 (7.6)	7.70 (7.5)	7.69-7.56		7.57 (7.5)	7.49-7.43		
NH-4	9.40	10.00	9.29		9.38	9.40		
	9.40	8 .99	9.22		9.14	8.99		
Me		3.87	3.87	3.89	3.89	3.93		3.85
		3.85	3.85			3.92		3.39
Ac	2.25, 2.25	2.23, 2.19	2.26, 2.24	2.18, 2.15	2.32, 2.27	2.33, 2.30	1.98, 1.92	2.21, 2.12
	2.11, 2.10	2.14, 2.13	2.19, 2.18	2.08, 2.05	2.25, 2.23	2.26, 2.23		2.08, 2.04
	2.07, 2.06	2.03, 1.97	2.14, 2.13	2.04, 2.04	2.21, 2.18	2.15, 2.14		2.04, 2.02
		1.94, 1.85	2.12, 2.11	1.94, 1.90	2.15, 2.13	2.11, 2.09		1.92, 1.90
			2.04, 1.99		2.11, 2.10	2.08, 2.05		
			1.97, 1.96		2.06, 2.04	2.04, 2.03		
			1.93, 1.85		2.03, 1.98	2.02, 2.00		
					1.93, 1.91	1.96, 1.90		
					1.89	1.88		
^{31}P	150.51	136.77	-7.61		135.25	-6.68	-5.48	
	149.67	134.28	-8.44		133.47	-8.31		

[a] The hydrogen chemical shifts in $CDCl_3$ (300 MHz, 298 K) are expressed relative to tetramethylsilane (0.00 ppm), and the vicinal hydrogen-hydrogen coupling constants in hertz are shown in parentheses. [b] The measurement was performed at 500 MHz (D_2O, 298 K, 50 mM ND_4CO_3), and the hydrogen chemical shifts are expressed relative to the HOD signal (4.80 ppm).

the reported data.[3f,4a] The H-3″ax resonance of the synthetic CMP-Neu5Ac (**2**) and its dimer **1** were observed as doublet of doublets of doublets. Since one of these couplings was a long range coupling between H-3″ax and the phosphorus atom, the spectrum suggested that the Neu5Ac-dimer was linked to the phosphorus atom by a β-configuration.[12]

In summary, we have developed a highly efficient synthetic route to CMP-Neu5Ac (**2**) and demonstrated its (**1**) application to the synthesis of the CMP-Neu5Ac-dimer (**1**) which had not previously been synthesized by reported methods. This route is not only concise and high yielding but it avoids unfavorable side reactions during large scale synthesis and decomposition of the synthetic intermediates. Although CMP-Neu5Ac synthetase has become increasingly available due to cloning techniques, preparation of CMP-Neu5Ac analogues has been limited due to the relatively high substrate specificity of CMP-Neu5Ac synthetase. Therefore, this processes promises to lead to the synthesis of other novel CMP-Neu5Ac analogues which had previously been unavailable.

Experimental Section

2-Cyanoethyl *2′,3′-O,N*^{4}-Triacetylcytidin-5′-yl *N,N*-Diisopropylphosphoramidite (4). To a solution of triacetyl cytidine (**3**) (3.5 g, 9.66 mmol), *N,N,N′,N′*-tetraisopropylphosphorodiamidite (4.37 g, 14.5 mmol), and diisopropylamine (1.95 g, 19.3 mmol) in dry MeCN (92 mL)-DMF (10 mL) was added 1*H*-tetrazole (1.35 g, 19.3 mmol) at 0 °C, and the mixture was then stirred for 1 h at room temperature. The mixture was diluted with EtOAc and washed with $NaHCO_3$ solution. After being dried with $MgSO_4$, the mixture was concentrated *in vacuo*. Purification of the residue by flash column chromatography (5% triethylamine in ethyl acetate) afforded **4** (3.9 g, 72%) as a diastereomeric mixture (1:1):R_f 0.6 (MeOH:EtOAc = 1:19); HRMS calcd for $C_{24}H_{37}O_9N_5P$ (M + H$^+$) 570.2329, found 570.2348.

2-Cyanoethyl *2′,3′-O,N*^{4}-Triacetylcytidine-5′-yl Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosid-2″-yl Phosphite (6). The pentaacetyl-Neu5Ac **5** (20.8 mg, 41.5 μmol) and cytidine 5′-*O*-amidite **4** (70 mg, 125 μmol) were separately dried by coevaporating twice with dry toluene. They were then combined in freshly prepared dry MeCN (0.5 mL). To this mixture was added 1*H*-tetrazole (10 mg, 145 μmol) at −40 °C under an argon atmosphere. After 5 min, the ice bath was removed. The mixture was further stirred for 30 min at room temperature, and then the mixture was diluted with EtOAc. The organic phase was washed with $NaHCO_3$ solution, dried with $MgSO_4$, and concentrated *in vacuo* at 40 °C. Purification of the residue by gel permeation column chromatography twice (Sephadex LH-20, i.d. 2 cm × 60 cm, MeOH,) afforded phosphite **6** (33 mg, 83%, 1.6:1 diastereomeric mixture): R_f 0.4 (MeOH:EtOAc = 1:9); HRMS calcd for $C_{38}H_{51}O_{22}N_5P$ (M + H$^+$) 960.2763, found 960.2809.

2-Cyanoethyl *2′,3′-O,N*^{4}-Triacetylcytidin-5′-yl Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosid-2″-yl Phosphate (7). To a solution of phosphite **6** (29 mg, 22.2 μmol, 1.6:1 diastereomeric mixture) in MeCN (0.1 mL) was added *t*-BuOOH (2.5 M in toluene, 88 μL), and the mixture was stirred at room temperature. After 30 min, the mixture was diluted with EtOAc and washed with $NaHCO_3$ solution. After drying with $MgSO_4$, the organic phase was concentrated *in vacuo* at 40 °C. Purification of the residue by gel permeation column chromatography (Sephadex LH-20, i.d. 2 cm × 60 cm, CH_2Cl_2) afforded phosphate **7** (26 mg, 90%, 1.6:1 diastereomeric mixture): R_f 0.3 (MeOH:EtOAc = 1:9); HRMS calcd for $C_{38}H_{51}O_{23}N_5P$ (M + H$^+$) 976.2713, found 976.2743.

CMP-Neu5Ac (2). To a solution of protected CMP-Neu5Ac **7** (73 mg, 75 μmol) in THF (1 mL) was added 1,8-diazabicyclo-[5.4.0]-7-undecene (13.6 mg, 90 μmol), and the mixture was stirred at room temperature. After 5 min, NaOMe (40 mg, 746 μmol) and MeOH−H_2O (0.7−1.4 mL) were added to this mixture. After 12 h, the mixture was lyophilized. Purification of the residue by gel permeation column chromatography (Sephadex G-15, i.d. 3 cm × 100 cm, water, 4 °C) afforded CMP-Neu5Ac **2** (33 mg 69%). The ^1H NMR spectrum of the synthetic CMP-Neu5Ac was in good agreement with the reported data:[3f,4a] ^{31}P NMR (D_2O, H_3PO_4 = 0.00 ppm) δ −4.43 ppm; HRMS calcd for $C_{20}H_{29}O_{16}N_4PNa_3$ (M + Na$^+$) 681.1010, found 681.1046.

OctaacetylNeu5Ac-dimer (10). To a solution of Neu5Ac dimer thioglycoside **9** (50 mg, 21 μmol) in acetone−H_2O (20:1, 0.5 mL) was added *N*-bromosuccinimide (9.5 mg, 53 μmol) at 0 °C. The mixture was stirred for 30 min at room temperature.

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The mixture was diluted with EtOAc and washed with $NaHCO_3$ solution. After being dried with $MgSO_4$, the organic phase was concentrated *in vacuo* . Purification of the residue by flash column chromatography (MeOH:EtOAc = 1:19) afforded **10** (16 mg, 89%): R_f 0.7 (MeOH:EtOAc = 1:9, 2 development); HRMS calcd for $C_{35}H_{49}O_{22}N_2$ ($M + H^+$) 849.2777, found 849.2815.

2-Cyanoethyl *2′,3′-O,N⁴*-Triacetylcytidin-5′-yl Neu5Ac-dimer-2″-yl Phosphite (11). The octaacetyl-Neu5Ac-dimer **10** (35 mg, 41 μmol) and cytidine 5′-*O*-amidite **4** (179 mg, 318 μmol) were separately dried by coevaporating twice with dry toluene. They were then combined in freshly prepared dry MeCN (0.8 mL), and to this mixture was added 1*H*-tetrazole (22 mg, 318 μmol) at - 40 °C under an argon atmosphere. After 10 min, the ice bath was removed. The mixture was further stirred for 30 min at room temperature, and then the mixture was diluted with EtOAc. The organic phase was washed with $NaHCO_3$ solution, dried with $MgSO_4$, and concentrated *in vacuo* at 40 °C. Purification of the residue by gel permeation column chromatography (Sephadex LH-20, i.d. 2 cm × 60 cm, MeOH) afforded phosphite **11** (46 mg, 85%, 1:1 diastereomeric mixture): R_f 0.3 (MeOH:EtOAc = 1:9, 2 development);HRMS calcd for $C_{53}H_{70}O_{31}N_6P$ ($M + H^+$) 1317.3823, found 1317.3819.

2-Cyanoethyl *2′,3′-O,N⁴*-Triacetylcytidin-5′-yl Neu5Ac-dimer-2″-yl Phosphate (12). To a solution of phosphite **11** (24 mg, 18.3 μmol, 1:1 diastereomeric mixture) in MeCN (0.5 mL) was added t-BuOOH (2.5 M in toluene, 73 μL), and the mixture was stirred at room temperature. After 30 min, the mixture was diluted with EtOAc and washed with $NaHCO_3$ solution. After being dried with $MgSO_4$, the organic phase was concentrated *in vacuo* at 40 °C. Purification of the residue by gel permeation column chromatography twice (Sephadex LH-20, i.d. 2 cm × 60 cm, CH_2Cl_2) afforded phosphate **12** (17.5 mg, 73%, 1.5:1 diastereomeric mixture): R_f 0.3 (MeOH:EtOAc = 1:9, 2 development); HRMS calcd for $C_{53}H_{70}O_{32}N_6P$ ($M + H^+$) 1333.3772, found 1333.3788.

CMP-Neu5Ac-dimer (1). To a solution of protected CMP-Neu5Ac-dimer **12** (22 mg, 17 μmol) in THF (0.4 mL) was added 1,8-diazabicyclo[5.4.0]-7-undecene (3 mg, 20 μmol), and the mixture was then stirred at room temperature. After 5 min, NaOMe (22 mg, 407 μmol) and MeOH−H_2O (0.2−0.4 mL) were added to this mixture. After 12 h, the mixture was lyophilized. Purification of the residue by gel permeation column chromatography (Sephadex G-15, i.d. 2 cm × 60 cm, 1 mM NH_4OH, 4 °C) afforded CMP-Neu5Ac-dimer (**1**) (9 mg, 56%); HRMS calcd for $C_{31}H_{45}O_{24}N_5PNa_4$ ($M+Na^+$) 994.1784, found 994.1825.

Methyl α-D-Neu5Ac-dimer (13): $[\alpha]^{25}_D$ −35.2° (*c* 0.75, $CHCl_3$); HRMS calcd for $C_{36}H_{51}O_{22}N_2$ ($M + H^+$) 863.2934, found 863.2974.

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Supporting Information Available: Copies of ^1H NMR spectra of **1**, **2**, **6**, **7**, **11**, and **12**, HMBC spectra of **13**, and ^{13}C NMR data for **4**, **6**, **7**, and **10**−**13** (9 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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